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January 15th, 2026

REVIEW
of the doctoral dissertation for the degree of doctor of medical sciences and health sciences in the discipline of health sciences

Candidate: Mariam Ibrahim, MSc
Title: Intergenerational and transgenerational effects of epigenetic factors applied in early developmental stages – insights from *in ovo* model
Supervisor: dr hab. Katarzyna Stadnicka, Wydział Nauk o Zdrowiu, Uniwersytet Mikołaja Kopernika w Toruniu, Collegium Medicum im. L. Rydygiera w Bydgoszczy

Introduction

This dissertation investigates how early-life exposure to bioactive compounds (a commercial synbiotic, PoultryStar®, and choline) administered *in ovo* to the developing embryo can shape intergenerational (F2) and transgenerational (F3–F4) molecular phenotypes across somatic and germline tissues. It addresses a current problem in epigenetic research — how dietary bioactive ingredients or other potential epigenetic factors affecting prenatal development can imprint gene regulation, and whether such changes persist beyond directly exposed individuals. The core novelty lies in (i) deploying a rigorously controlled *in ovo* system to minimize maternal confounding exposures, (ii) following multiple generations (F1–F4), and (iii) combining transcriptomics across four tissues (cecal tonsils, cecal mucosa, embryonic blood, and gonads) with methylome profiling in gonads.

The thesis argues that the chicken embryo serves as a powerful platform for investigating the inheritance of epigenetic signals. This is due to its oviparity, short generation time, and status as an ethically viable vertebrate model system. Additionally, the chicken possesses a well-annotated genome that shows substantial conservation of genes and chromosomal segments with humans. In terms of non-coding conservation, approximately 38% of chicken CpG islands — often associated with regulatory elements — are conserved in the human genome. Therefore, applying an *in ovo* cross-generational model at very early preclinical stages (prior to transitioning to more relevant but less accessible mammalian or human studies) offers meaningful insights into potential epigenetic effects induced during embryonic

development. Importantly, this model allows for the distinction between somatic and germline epigenetic effects under controlled conditions — an outcome that is challenging to achieve in human or mammalian systems.

Further, the thesis include primordial germ cells (PGCs) as both biological subject and model tool, demonstrating marker usefulness of PGCs after short- and long-term freezing. The thesis suggest a valuable study platform with translational potential for metabolic and immune programming at early stages, prior to shifting to more clinically relevant, and more challenging mammalian models.

Dissertation structure and compliance with title

The dissertation is presented as a monothematic cycle comprising for published Papers (I-IV) and one drafted original research manuscript (V), namely: Paper I (mini-review; Frontiers in Cell & Developmental Biology, 2025, IF 4.6) providing the theoretical framework for the dissertation and discussing PGCs as a model for nutriepigenetic–metabolic research; Paper II (Genes, 2024, IF 2.8) on PGC cryopreservation; Paper III (Int. J. Mol. Sci., 2025, IF 4.9) mapping inter-/transgenerational effects in somatic tissues of cecal tonsils and cecal mucosa; Paper IV (Scientific Reports, 2025, IF 3.9) describing transgenerational effects in gonads, involving methylome analysis; Manuscript V (draft) on effects in embryonic circulating blood, which carries future gametes colonizing gonads (in F3–F4 generations).

The dissertation logically synthesizes the monothematic cycle of studies. The coherence of monothematic cycle is clear and very well justified. The thesis consists of typical set of chapters: Introduction, Aims and hypothesis, Thematic coherence of the publications, Background, Methodology, Results section containing highlights of results for each of the articles, Discussion, Limitations, Conclusions, and Perspectives, followed by the annexed papers and references. The content of the thesis is faithful to the title, which accurately reflects the general scope (inter- & transgenerational epigenetic effects; early developmental application; *in ovo* model).

Regarding the editorial quality, the text is generally well edited using consistent terminology across the text, for example SYN/SYNCH for all the treatments with synbiotic and synbiotic combined with choline; F1–F4 for consecutive generations; HH (for Hamburger-Hamilton embryonic developmental staging), and includes comprehensive abbreviations provided in the beginning of the thesis. The methods are outlined with sufficient attention to the details (quality control pipelines, reference genomes, thresholds), and maintains figures/tables legibility.

Some minor stylistic repetition occurs when article restate points already mentioned in the synopsis. However, this can be considered as common in thesis formats based on publications cycle, and doesn't affect the overall clarity of the thesis or understanding.

Assessment of methodology

The study design is well described and justified. The choice of *in ovo* study model and exposure to epigenetic stimuli at 12th embryonic developmental day is well justified. The candidate used the earlier, in-house developed methodology for embryo stimulation, with robust replication through high egg numbers. Ethical approvals and husbandry are described with care in the published papers.

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Dose optimization experiments were performed prior to main trials and identified 2 mg synbiotic + 0.25 mg choline as a combination ensuring unaffected hatchability of the experimental animals and ensuring internal validity for the main experiment across generations.

The selected tissue panel is appropriately chosen to study immune- related effects (cecal mucosa/tonsils), and germline lineage (gonads, gametes precursors and circulating embryonic blood). The molecular analysis pipelines employed in the thesis are clearly defined, and aligned with current standards. Differential expression analysis was conducted applying rigorous p-values and tissue-specific thresholds to identify significant genes. Functional enrichment was carried out using clusterProfiler, SRplot, and Pathview, with expression trends further validated via RT-qPCR on selected genes for each tissue. Integration of gene expression and methylation data was achieved using orthology-based identifiers to link differentially expressed genes (DEGs) with differentially methylated genes (DMGs).

Overall, the applied bioinformatic pipelines are transparent, and appropriate for the study's aims. The thesis publications also includes documentation of data deposition for tissue samples, ensuring data accessibility and reproducibility. A minor limitation is that some bioinformatic versioning differs slightly across tissues (GRCg6a vs GRCg7b), but this is disclosed and does not compromise conclusions given within-tissue comparisons against matched controls per generation.

Assessment of research results

The key research findings revealed distinct and generation-specific transcriptomic and epigenetic responses across tissues, treatments, and generations, with patterns that support transgenerational plasticity. In the cecal tonsils, differential gene expression (DEG) analysis showed a drop in DEG counts in the F2 generation, followed by a clear increase in F3. This pattern is claimed to be consistent with a “generational skipping” phenomenon, observed in other transgenerational studies. Functional enrichment analyses pointed to pathways involved in metabolism, immune signaling, and homeostasis. In the cecal mucosa tissue, the F2 generation showed a stronger intergenerational transcriptional response. Enriched KEGG pathways were predominantly metabolic.

A limited gene overlap was observed across generations and groups. Could candidate explain this sparse overlap?

The embryonic blood transcriptome was tested in F3 and F4 generations, and revealed enriched expression of genes involved in ribosomal and protein biosynthesis pathways in F3. In the gonads, the highest transcriptional responses were observed after the treatment of combined synbiotic with choline. The methylome analysis in the gonads revealed a higher number of differentially methylated regions (DMRs) and loci (DMLs) in F3 compared to F2. The repeated prenatal exposure to synbiotic with choline led to a broader methylation changes than single treatment of synbiotic with choline. The candidate managed interpretation and presentation of these complex results with a high scientific maturity. The mapping of GO/KEGG terms in attempt to explain mechanisms of synbiotic and choline effects was careful and did not overreach. Importantly, the candidate acknowledged the limitations of her work, noting that epigenetic assays beyond the gonads tissue still need to be conducted. She also recognized that the causal relationships proposed in the thesis are suggestive rather than conclusive in non-gonadal tissues. Additionally, she pointed out that, due to cost constraints,

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sampling of gonads and embryonic blood was not carried out consistently across all generations, which made it harder to track changes in detail over time.

Altogether, the dissertation supports hypothesis that prenatal stimuli can pose intergenerational (F2) and transgenerational (F3–F4) molecular consequences, that tissue context (immune - gut versus germline) strongly shapes the observed effects, and that PGCs in the *in ovo* model provide a practical and meaningful tool for germline studies.

Terms such as "generational skipping," "washout effect," and "silent carrier" are used in the discussion. Could the candidate provide literature-based justification for these terms and clarify their relevance to the observed data?

Summary and recommendation

This dissertation presents a rigorous, original, and methodologically robust multigenerational investigation, demonstrating that epigenetic factors can induce specific intergenerational and transgenerational changes. It offers significant scientific insights using a well-controlled *in ovo* model.

Given its scientific quality, logical structure, publication record, technical depth and candidate's contributions, I find that the dissertation meets the conditions specified for the award of the doctoral degree in accordance with the conditions specified in Article 187, Sections 1-4 of the Act of July 20, 2018, on Higher Education and Science (consolidated text, Journal of Laws of 2018, item 1668). In connection with the above, I submit to the Disciplinary Council of Health Sciences at Nicolaus Copernicus University in Toruń, Collegium Medicum in Bydgoszcz, a motion to admit to the further stages of doctoral proceedings. I additionally recommend that the doctoral dissertation be considered for distinction by the Disciplinary Council of Health Sciences, provided that it complies with the formal criteria set forth in the applicable regulations. I additionally recommend that the doctoral dissertation be considered for distinction by the Disciplinary Council of Health Sciences, provided that it complies with the formal criteria set forth in the applicable regulations.

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